ANTIGENIC RELATIONSHIPS AMONG FLOC-FORMING *PSEUDOMONADACEAE*[1]

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Abstract. A number of cross-reactions were observed between the antigens of floc-forming pseudomonads. Immunodiffusion tests of trichloroacetic acid extracts showed that antigens are shared by some *Zoogloea* spp. and *Pseudomonas* spp., yet other *Zoogloea* and *Pseudomonas* spp. lack these antigens. One of the antigens was also found in *Gluconobacter*. This demonstration of a close antigenic relationship between some, but not all, members of the 3 genera must be weighed against the limited number of characteristics which establish the generic distinction.

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Members of the genera *Zoogloea*, *Gluconobacter*, and *Pseudomonas* share many of the same distinctive characteristics, and studies of ecological significance (Crabtree and McCoy 1967, Friedman and Dugan 1968, Friedman *et al* 1968, Dugan 1975) have been handicapped by difficulties in identification. These bacteria are all gram-negative, polarly-flagellated, aerobic rods, and are frequently isolated from aquatic habitats where they grow as flocculent aggregates of cells rather than dispersed. They are nonfermentative, catalase-positive organisms which produce varying amounts of extracellular polymer that has been implicated in the bio-flocculation process (Friedman *et al* 1969). Interest in these organisms is related to the ecological significance of the flocculation phenomenon in removal of organic and mineral water pollutants (Dugan and Pickrum 1972). Many of these bacteria are indigenous to aerobic waste treatment systems and have high adsorptive capabilities in addition to strong hydrolytic and oxidative capacities (Joyce and Dugan 1970).

There are few reports concerning antigenic relationships among the *Pseudomonadaceae* other than for the genus *Pseudomonas*. An exception is McIntosh (1962), who studied antigenic analysis of organisms attributed to the genus *Acetobacter* using agglutination and tube precipitation tests. He reported that *A. suboxydans* (*Gluconobacter*) strains constituted a distinguishable serologic group, unrelated to *A. rancens*. Other species were not included in the study. In view of the difficulties in identifying pseudomonad bacteria, we have examined the serologic reactions of examples in 3 of the genera mentioned above.

MATERIALS AND METHODS

Organisms. Pseudomonads included in this study were *Zoogloea ramigera* 115 (ATCC 25935), *Z. filipendula* P84, *Z. ramigera* 1-16-M (ATCC 19623), *P. denitrificans* (ATCC 13867), *Gluconobacter oxydans*, subsp. *oxydans* (ATCC 9433), *G. oxydans* subsp. *suboxydans* (ATCC 621) and *Pseudomonas* species C3 which was isolated from raw water and previously described (Friedman and Dugan 1968).

Preparation of Antigens. Cultures were grown for 2 days at 26°C in a 12-liter fermenter containing trypticase soy broth (TSB) (Becton, Dickinson, Cockeysville, MD) except that mannitol-yeast extract-peptone broth was used for growing the *Gluconobacter* spp. The cells were collected by centrifugation, using the KSB continuous-flow apparatus (Sorvall, RC2B Centrifuge) and washed twice in saline by the same procedure. The antigens were extracted from whole cells with 0.25N trichloroacetic acid (TCA) by the method of Van Eeden (1967) except that the TCA soluble extracts were dialyzed against 3 changes of phosphate-buffered saline (pH 7.4) at 4°C. An extract of TSB was prepared in the same way, as a control. Extracts were stored at 4°C with 0.02% sodium azide added as a preservative.

Antisera. Rabbits were immunized by injecting suspensions of the heat-killed organisms (4×10^9 cells) subcutaneously in Freud's incomplete adjuvant. The schedule consisted of

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2 injections a month apart. Some animals were injected a third time after a lapse of several months. Test sera were collected when trial bleedings produced good homologous reactions.

Immunodiffusion Tests. Antisera were examined by immunodiffusion against trichloroacetic acid extracts of the homologous and heterologous organisms. Some immunodiffusion tests were performed in Petri plates and some by the micro-modification (Wadsworth 1957). In either case, 1% Noble Agar was employed and 0.05% trypan blue was added for better visualization. Precipitation patterns were read at various times, but examples shown in the figures were photographed between 18 and 36 hours.

RESULTS

Antisera to 6 floc-forming pseudomonads, which produced strong precipitation reactions with homologous extracts, cross-reacted with extracts from certain other pseudomonads. Both of the *Zoogloea* spp. exhibited cross-reactions with *Pseudomonas* sp. C3 and one cross-reacted with *Gluconobacter* spp. These antisera did not cross-react with extracts of *P. denitrificans*. At least 6 cross-reactions of apparently different specificities were observed.

Immunodiffusion tests indicated the complexity of the cross-reaction (fig. 1a) and produced partial clarification. Some antisera against *Zoogloea ramigera* 115 defined an antigen which is identical with one of *Pseudomonas* sp. C3 and *Gluconobacter oxydans* subsp. *suboxydans* (fig. 1b). The reactions of antisera against *Pseudomonas* sp. C3 likewise demonstrated strong cross-reactions with *Z. ramigera* 115 (figs. 2a and 2b). The reaction of identity is clear in figure 2b. A different antiserum against C3 again exhibited cross-reactions with *Z. ramigera* 115 but also showed a reaction of identity with both *G. oxydans* subspecies (fig. 2c). The cross-reactions of anti-C3 with *Z. ramigera* 115 and *Z. ramigera* 1–16–M were confirmed with still another antiserum (fig. 2d), but a reaction between this serum and *Z. filipendula* P84 could not be demonstrated.

Evidence that antibody specificities in antisera raised to *Z. ramigera* 115 and *Pseudomonas* sp. C3 are identical is presented in figure 3, where the reactions of the 2 antisera with an extract of *Pseudomonas* sp. C3 were reactions of identity. A similar experiment, using an extract of *Z. ramigera* 115 as the antigen, confirmed this identity.

Using an antiserum to *Z. ramigera* 1–16–M, reactions of identity were observed with *Z. ramigera* 115 and *Pseudomonas* sp. C3, but were weak and could not be clearly photographed. Antisera to *Z. filipendula* P84 yield cross-reactions with *Pseudomonas* sp. C3 and *Z. rami-*

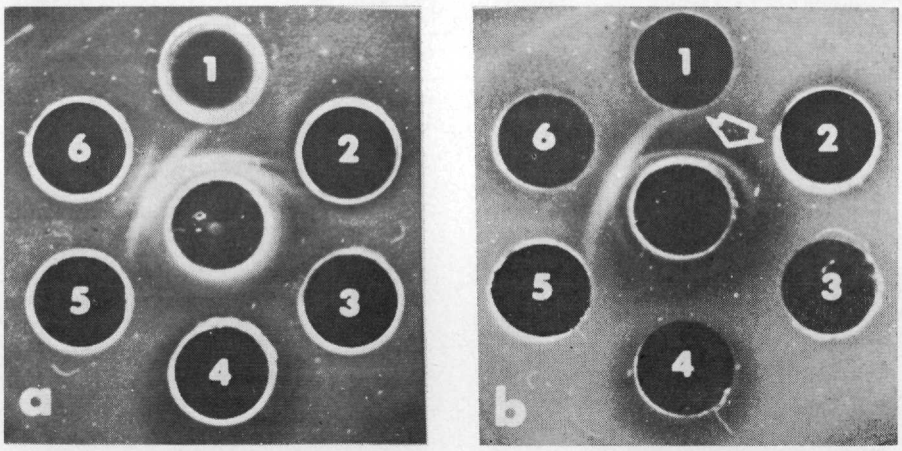


FIGURE 1. Cross-reactions between antisera to *Z. ramigera* 115 (center well) and other pseudomonads: *Pseudomonas* sp. C3 (1), *Gluconobacter oxydans* subsp. *suboxydans* (2), *Gluconobacter oxydans* subsp. *oxydans* (3), *Z. filipendula* P84 (4), *Z. ramigera* 1–16–M (5), and *Z. ramigera* 115 (6). Fig. 1a shows a multispecific serum, Fig. 1b shows a reaction of identity (arrow) between *Z. ramigera* 115 (6), *Pseudomonas* C3 (1), *Z. ramigera* 1–16–M (5) and *Gluconobacter oxydans* subsp. *suboxydans* (2).

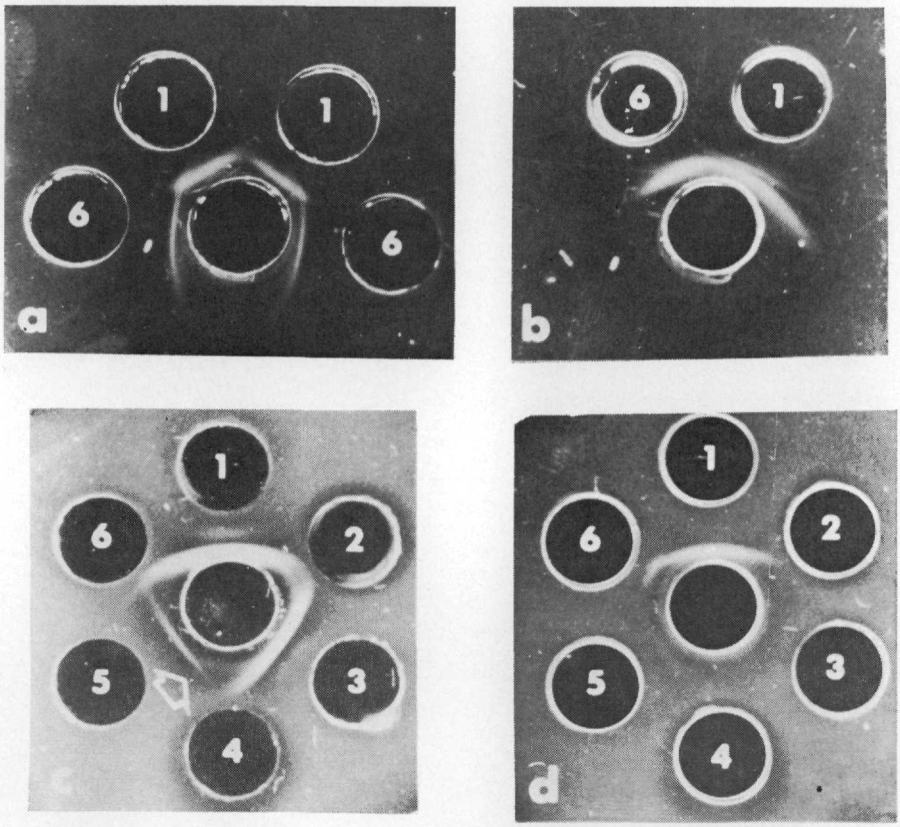


FIGURE 2. Cross-reactions between antisera to *Pseudomonas* sp. C3 (center well) and extracts of other pseudomonads. Fig. 2a and 2b show reactions with *Pseudomonas* sp. (1) and *Z. ramigera* 115 (6). Fig. 2c shows reactions of identity with *Z. ramigera* 115 (6), *G. oxydans* subsp. *oxydans* (2) and *G. oxydans* subsp. *suboxydans* (3). Also present (arrow) is a reaction of partial identity with *Zoogloea filipendula* P84 (4) and *Zoogloea ramigera* 1–16–M (5). Fig. 2d shows a reaction of identity between *Pseudomonas* sp. C3 (1), *Z. ramigera* 115 (6) and *Z. ramigera* 1–16–M (5) with a different preparation of antiserum. Although the latter reaction (5) is faintly visible in the photo, it was clearly seen in the gel.

gera 1–16–M, but not with *Z. ramigera* 115, thus appearing to involve a different antigen.

The cross-reaction between the *G. oxydans* subsp. *suboxydans* and *Z. ramigera* 115 is shown in figure 4a. Clearly, 2 or more antigens relate these organisms. Whether one of these is the same specificity as the one observed in antisera to *Z. ramigera* 115 and *Pseudomonas* sp. C3 has not been determined. The *Gluconobacter* spp. shared a different antigen which was not demonstrated in either *Z. ramigera* 115 or *Z. filipendula* P84, (fig. 4b). A list of the antigenic relationships observed is presented in table 1.

DISCUSSION

A variety of antigens have been demonstrated in floc-forming pseudomonads which cross the lines of established genera. No single antigen defines a species or genus and the presence of a variety of carbohydrate antigens is suggested. At least 3 different antigenic determinants are shared by *Zoogloea ramigera* 115 and *Pseudomonas* sp. C3, indicating a close relationship between these organisms. One of these determinants was also found in *Z. ramigera* 1–16–M, but none were demonstrated in *Z. filipendula* P84, or in *Pseudomonas denitrificans*.

Table 1 shows that a number of in-

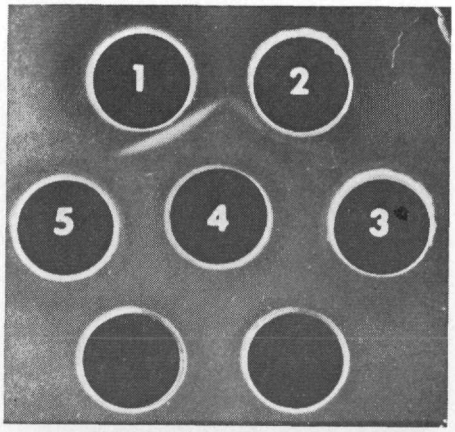


FIGURE 3. Identity of the major antibody specificities in antisera to *Pseudomonas* C3 (1) and *Z. ramigera* 115 (2) was shown by testing against *Pseudomonas* sp. C3 extract (4). Wells 3 and 5 contained antiserum to *Z. filipendula* P84 and the two bottom wells were empty.

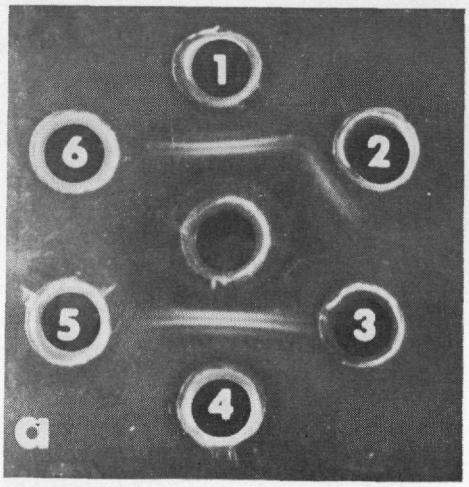
TABLE 1

Reactions of Identity Observed with Heterologous Species

Antiserum Against:	Extract From:
Z. ramigera 115	*Pseudomonas* sp. C3 *G. oxydans* subsp. *suboxydans* *Z. ramigera* 1–16–M
Z. ramigera 1–16–M	*Pseudomonas* sp. C3
Pseudomonas sp. C3	*Z. ramigera* 115 and 1–16–M *G. oxydans* subsp. *suboxydans* *G. oxydans* subsp. *oxydans*
Z. filipendula P 84	*Z. ramigera* 1–16–M *Pseudomonas* sp. C3
G. oxydans subsp. *suboxydans*	*Z. ramigera* 115 *G. oxydans* subsp. *oxydans*
G. oxydans subsp. *oxydans*	*Z. filipendula* P84 *G. oxydans* subsp. *suboxydans*

teresting relationships were demonstrated and that several different antigens appear to be shared across generally accepted taxonomic lines. This situation is reminiscent of the occurrence of the somatic *O* antigens in enteric bacteria. In these organisms, the same antigen may occur in different taxa and it is necessary to establish a pattern of antigens in order to place an isolate in a particular group.

From the results presented, it appears that the organism presently classified as *Zoogloea ramigera* does not match closely with *Zoogloea filipendula* on a serologic basis. Likewise, the serologic pattern of *Pseudomonas* sp. C3 does not match that of *P. denitrificans*. It also appears that *Z. ramigera* 115 has a greater antigenic



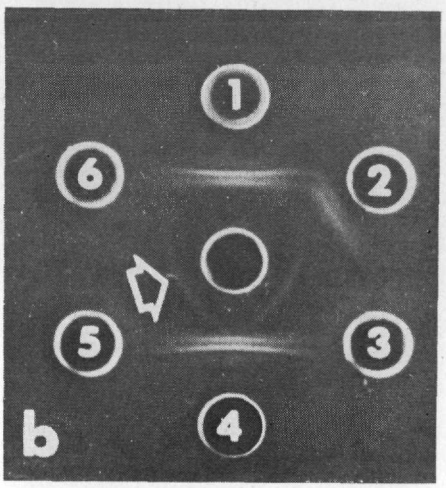


FIGURE 4. Cross-reactions between antisera to *G. oxydans* subsp. *suboxydans* (center well) and extracts of other pseudomonads. Fig. 4a shows double reactions of identity between *G. oxydans* subsp. *suboxydans* (1 and 4), and *Z. ramigera* 115 (2) while *Pseudomonas* sp. C3 (3), *Z. filipendula* P84 (5) and *Z. ramigera* 1–16–M (6) did not react. Fig. 4b shows a different reaction of identity (arrow) between *G. oxydans* subsp. *oxydans* (3 and 5) and *G. oxydans* subsp. *suboxydans* (1 and 4) which was not shared with *Z. ramigera* 115 (2) or *Z. filipendula* P84 (6).

relatedness to *G. oxydans* subsp. *suboxydans* than to either *Z. filipendula* or *Z. ramigera* 1–16–M. These data and other observations (Dugan 1975) suggest that *Z. ramigera* 115 and *Z. ramigera* 1–16–M are distinctly different serotypes.

No claims are made relative to the number of different cross-reacting specificities which are present in the organisms which were studied. Antisera prepared in this investigation, however, have demonstrated several shared antigens and these may be found useful in defining groups of organisms. The mere fact that an antiserum failed to detect a particular antigen in extracts from an organism does not preclude its existence, since the response of the rabbit and the amount of antigen in the extract are important variables which may result in such failures even though the antigen is present. Much future work will be needed to firmly establish the distribution of these antigens. Nevertheless, the findings presented here suggest that distinctive antigens can be demonstrated in the pseudomonads which may permit the application of serotyping techniques for identification. In view of the limited number of characteristics which distinguish some pseudomonads (Crabtree and McCoy 1974, DeLay and Flateur 1974, Dugan 1974), such techniques might be useful.

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